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Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening

Piotr Strus

HESA Histology and Embryology Students Association at the Department for Histology and Embryology Centre for Biostructure Research, Warsaw Medical University, Poland

Kamil Lisiecki

Faculty of Chemistry, University of Warsaw, Pasteura 1, Warsaw 02-093, Poland

Zbigniew Czarnocki

Faculty of Chemistry, University of Warsaw, Pasteura 1, Warsaw 02-093, Poland

Izabela Mlynarczuk-Bialy*

Department for Histology and Embryology Centre for Biostructure Research, Warsaw Medical University, Poland

Lukasz P Bialy

Department for Histology and Embryology Centre for Biostructure Research, Warsaw Medical University, Poland

* Corresponding author: imlynarczuk@wum.edu.pl, Chalubinskiego 5, 02-004 Warszawa, Poland

Abstract: The systemic treatment of advanced cancer, where local surgery is ineffective, still offers numerous difficulties due to therapy resistance, side effects and relapse. Thus, novel therapeutics are ongoing. The scaffolds for novel drugs often derive from naturally occurring substances. One of such example is podophyllotoxin – a plant derived substance. Podophyllotoxin itself is indicated in local anticancer treatment and due to its' substantial toxicity is not recommend for systemic therapy. However, its analogues like etoposide is an element of systemic anticancer therapy administered in many malignancies. Limiting for etoposide is bone marrow depression and secondary leukemia induced in susceptible individuals. Thus, less toxic and more effective substances are needed for cancer therapy. Due to complexity of podophyllotoxin molecule it's scaffold is nowadays intensively studied as a source for novel therapeutic substances. Within this project our team aimed to modify the molecule of podophyllotoxin to obtain novel derivatives that were screened for anticancer potential. Furthermore, one of new compound was conjugate of podophyllotoxin and benzothiazole. Benzothiazole is widely used in research because of antitumor, antibacterial, anticonvulsant, anti-inflammatory and other activities of its derivatives. The obtained derivates turned out to be less toxic for normal fibroblasts in comparison to parental podophyllotoxin.
Key words: podophyllotoxin, benzothiazole, cancer, cell viability

1. Introduction

A number of studies and many clinical examples demonstrate that the treatment of cancer often encounters numerous difficulties. The lack of therapy response, relapse, or even toxic side effects are a few of such examples [1, 2]. Moreover, in developed countries, the prevalence of cancer shows increasing tendency and application of cancer medicals makes its treatment one of the most costly [1, 2]. In advanced stages of malignant disease the existing treatment remains often ineffective or relapse is diagnosed [1, 2]. Hence, to overcome these difficulties new drugs that are effective, specific, less toxic and relatively cheap are ongoing.

Thus, the ideal anti-cancer drug – would be one that combined two features: nontoxic for normal cells and effectiveness for tumor cells [3, 4]. It also should be cost-effective and have uncomplicated application.

Over last years, natural products continued to play a highly significant role in the drug discovery and development process. Thus in the area of anticancer drugs, according to published data including 25-year period of drug development, 47% of all approved antitumor drugs worldwide were either natural products or directly derived therefrom [5].

Our team was inspired by the ideas above. Therefore, we designed and synthesized derivatives of podophyllotoxin (PTOX) which is the anti-cancer, plant-derived drug [6, 7]. PTOX belongs to the class of aryltetralinlactone cyclolignans and it is purer and more stable form of podophyllin [8-14].

The crude *Podophyllum peltatum* plant extract is named podophyllin. However, podophyllin as a mixture of different active substances contains little active compound and numerous harmful ingredients of high toxicity, thus does not comply with the WHO guidelines for plant derived treatments and is not recommended for clinical treatment protocols [15].

The purified form of podophyllin-derived active compound is named podophyllotoxin. This polycyclic compound was first isolated in 1880 from the *Podophyllum peltatum* species. However, there are few other species, such as *Berberidaceae*, *Apocynaceae*, *Polygalaeae*, *Apiaceae*, *Linaceae* and other, which contain PTOX and analogs. PTOX contains five rings, which are methylenedioxy, two tetrahydronaphthalene, lactone and aryl rings (Scheme 1). PTOX is common and the most effective cure for anogenital warts; especially for condyloma acuminatum caused by human papilloma virus (HPV) [16-18].

The exact mechanism of antineoplastic action of PTOX is unknown. However, it has been shown that PTOX due to binding tubulin, a subunit of microtubules, prevents the polymerization of tubulin into microtubules. Affinity and site of binding is similar to colchicine, well-known plant-derived (*Colchicum autumnale*) drug with similar mechanism [19], although PTOX binds more rapidly and reversibly whereas colchicine bind irreversibly. Thereby, this mechanism could include the cell cycle arrest at mitosis and impede the formation of the mitotic-spindle [7]. Interestingly, PTOX competitively inhibits the binding of colchicine [20]. As mentioned above, PTOX alone is used in a local treatment of anogenital warts [21, 22]. Clinical results with systemic application of PTOX were disappointing due to severe gastrointestinal side effects [23], therefore PTOX won't be approved for systemic treatment. However, continuous efforts concerning the synthesis of PTOX analogues led to the discovery of new anticancer drugs. For example its derivative, etoposide is currently used in the clinic for the treatment of a variety of malignancies including lung and testicular cancers, glioblastoma multiforme, lymphoma and nonlymphocytic leukemia [24]. Teniposide another PTOX derivate, is applied for the treatment of childhood acute lymphocytic leukemia [24]. In contrast to the parent podophyllotoxin, which binds to tubulin and inhibits microtubule assembly, etoposide has a distinct mechanism of action [25]. In fact, molecules such as etoposide, amsacrine, and mitoxantrone are topoisomerase II inhibitors that induce cell death by enhancing topoisomerase II-mediated DNA cleavage through stabilization of the transient DNA/topoisomerase II cleavage complex [25, 26]. Like numerous anticancer drugs, also etoposide is not free of toxic side effects. Bone marrow depression is a serious, dose-limiting side effect diagnosed in patients receiving etoposide [27]. The use of effective doses of etoposide is also associated with an increased risk of secondary acute myelogenous leukemia. For this reason, there exist an urgent need for development of more potent analogues of podophyllotoxin characterized by less toxic side effects.

Within this study we aimed to modify the molecule of phodophyllotoxin by binding it with functional groups to obtain novel compounds with potential for systemic application.

Therefore, three new cyclolignans were synthesized using photocyclization or acid-catalyzed cyclization strategy [28, 29]. One of the new compounds, named KL3, is a conjugate of two molecules with anti-tumor activity, podophyllotoxin and benzothiazole. The analogues of benzothiazole and its derivates are widely used in pharmaceutical research, because of their biological and pharmacological properties. Benzothiazole is a class of heterocyclic compounds having 2 hetero atoms: sulphur and nitrogen [18]. Benzothiazole is a privileged bicyclic ring system with multiple applications. In the 1950s, 2-aminobenzothiazoles were intensively studied as central muscle relaxants. Several years later riluzole was found to interfere with glutamate neurotransmission (6-trifluoromethoxy-2-benzothiazolamine, PK-26124, RP-25279, Rilutek). After that benzothiazole derivatives have been also studied as anticancer agents [30]. The potential of benzothiazole and related compounds was examined in breast tumors, regardless of estrogen receptor status, and against ovarian, renal, lung, and colon cancer cells [31].

Derivatives of benzothiazole serve as scaffolds for experimental drug design without technical difficulties [32-38]. This versatile and unique compounds have received remarkable attention because of antitumor activity against breast (both estrogen receptor-positive and estrogen receptor-negative cell lines), ovarian, colon lung and renal cell lines and their interesting pharmacological activities, including anticonvulsant, analgesic, anti-tumor, antibacterial, antimicrobial, skeletal muscle relaxant and other activities such as: antidiabetic, anti-inflammatory, anticonvulsant, antiviral, antioxidant, antitubercular, antimalarial, antiasthmatic, antihelmintic, photosensitizing and diuretic [31, 39-51].

In 2016 we have developed methodology for the stereoselective synthesis of cyclolignans related to podophyllotoxin [28]. It involves the use of L-prolinol as a chiral auxiliary and continuous flow irradiation of a chiral atropo-

isomeric 1,2-bisbenzylidenesuccinate amide ester. Based on this discovery, a formal synthesis of (-)-podophyllotoxin and total synthesis of (+)-epigalcatin were completed [28, 29, 52].

New compounds should be soluble and stable in water solution. They need to have acceptable bioavailability, easily pass through cancer cells membrane and they should provide the evidence of high selectivity against cancer cells. An unsophisticated and cheap way to fast screening of new compounds is their incubation with selected cell lines in a cell culture. We can observe the effects of novel compounds on proliferation of cells, cell morphology and identify signs for degeneration using microscopic imaging as well as we can study cytotoxic/cytostatic effects within viability assays [53-55].

An assessment of cell survival is essential to test if compounds have cytotoxic/cytostatic effects. Cytotoxic effect results from direct harmful effect of examined compound on cells and cytostatic effect is a consequence of decreased cell proliferation that secondly may also lead to cell death. One of the best established viability assays is crystal violet decolorization assay (CVDA). In this method staining of attached, living cells with crystal violet dye, which binds to proteins and DNA, allow to detect the amount of remaining alive cells. Dead cells lose their adherence and by washing with PBS are eliminated from the population of cells, reducing the amount of bound crystal violet in a culture in comparison to the control, vehicle-treated group. This is a quick and if validated, adequate screening method that can be used for the estimation of cytostatic/cytotoxic effects after treatment with potential anticancer compounds [53-55]. Therefore, to estimate the number of living cells we used CVDA.

To improve our results and determine cytopathic effects of novel compounds we also used light microscope. Due to specification of our Juli-Stage (NanoEnTek) microscope we were able to verify proliferation and amount of cells and also take photographs of cells incubated with new compounds.

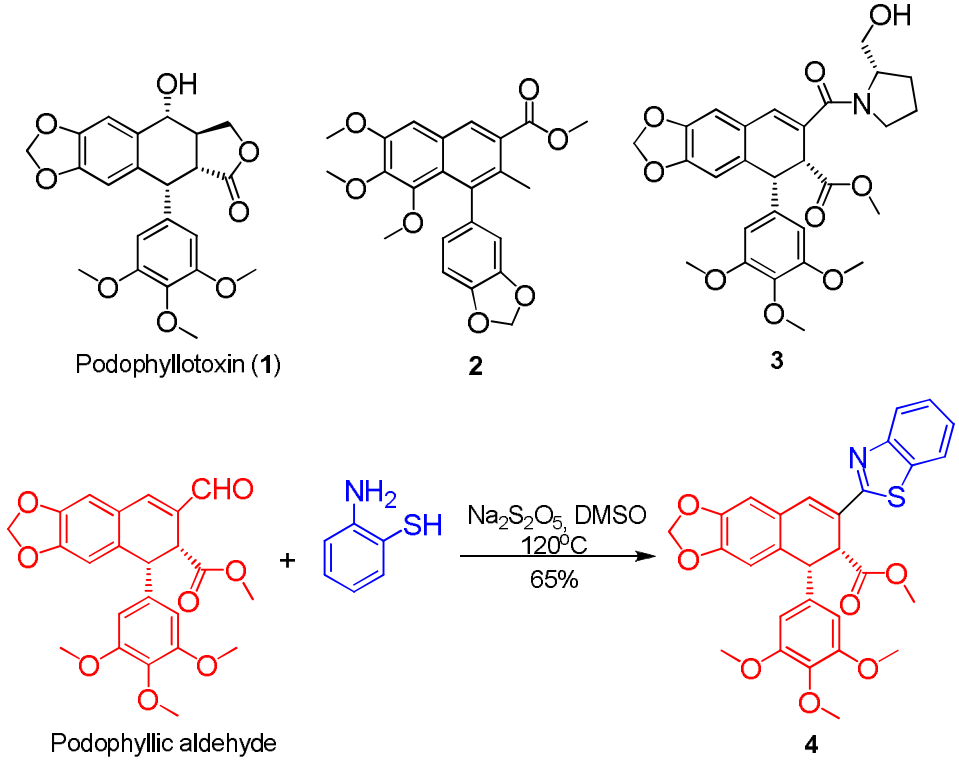
2. Results and discussion

2.1. Design of PTOX derivates

As shown in the available literature, cyclolignans are compounds with remarkable potential. However, most of derivatives are obtained by modifying natural compound (mainly podophyllotoxin). This does not allow for accessing other stereoisomers because some stereochemical features in the main carbon skeleton of podophyllotoxin cannot be changed during synthesis. What is more, the structural complexity of podophyllotoxin, derived from the presence of four stereogenic carbons in ring C has limited most of the structural activity relationship obtained by derivatization of the parent natural product rather than by the total synthesis. These features make it necessary to search for derivatives of PTOX with simplified structures, which can be obtained *via* short synthetic pathway from simple starting materials. Such are needed, since the therapeutic potential of PTOX and its derivatives is often limited by problems of drug resistance [56], hydrophobicity and low selectivity [8]. Although many methods of stereoselective synthesis of cyclolignans are known, their main drawback is the lack of generality and often high cost of synthesis. To meet this, we developed new methodology [57] based on the use of cheap and readily available starting materials such as simple aromatic aldehydes, succinic acid ester, and L-prolinol as a chiral auxiliary. The key step of the synthesis is photocyclization. It has been shown that this process occurs at much higher yield when carried out under flow conditions. This approach has an additional advantage, it allows for unlimited scale of synthesis, which is an important issue in the multi-step syntheses. In addition, avoiding the use of sophisticated organocatalysts or catalysts containing heavy metals, will be extremely important because of the elimination of the possibility of contamination with toxic metals. Our method is therefore extremely advantageous in the context of the synthesis of compounds with potential pharmaceutical application.

In this study, we investigated anticancer properties of three new derivatives of PTOX - **2**, **3**, **4**. Compound **2** is a derivative of 1-arylnaphthalene which poses anti-cancer activity [58]. Derivative **3** contains in its structure L-prolinol moiety which is a H-bond donor and its conformationally restricted main chain may improve receptor binding. Compound **4** is a conjugate of podophyllotoxin (red) and benzothiazole moiety (blue). In recent years, benzothiazoles have been recognized as promising pharmacophores with diverse biological properties including anti-cancer activity [47]. All of those

compounds are in good accordance with Lipinski's rule of five [59] – less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors and molecular mass less than 500 daltons or not much above. Compounds **2** and **3** were obtained by us previously [28, 29] and compound **4** was synthetized from podophyllic aldehyde (total synthesis of this compound was already reported by us [28]) and 2-amino-thiophenol (Scheme 1). The synthesis of presented compounds is highly efficient and starts from cheap and readily available substrates.



Scheme 1. Chemical structures of podophyllotoxin (**PTOX**), compounds 2 -**KL1** and 3 -**KL2** (top) and synthesis of compound 4 -**KL3** (bottom).

2.2. Cytotoxicity assay

We performed crystal violet viability assay to examine the sensitivity of tumor and non-tumor cells towards novel derivates in comparison to the parental one PTOX.

Compound KL1 did not induce any significant cytotoxic/cytostatic effect against HeLa, MDA-MB, DU145 and CFPAC. Also, in comparison to PTOX – a model toxic drug for NIH-3T3 cells, the novel KL1 compound was 100 times less toxic. Similar results were obtained for KL2 compound. The values of IC50 for this substance on all but one cell line (MCF7) was good above 140 μM and ranged from 140 μM to 490 μM (Table 1). In addition, in PC3 cell line the IC50 for KL1 was 48 μM. Also, non-tumor NIH-3T3 cells turned out to be 90 times less susceptible to the action to KL2 in comparison to the parental PTOX.

These results showed that compounds KL1 and KL2 by chemical modifications of parental PTOX exhibited lower cytotoxicity than PTOX itself. Moreover, both compounds turned out to be highly effective in induction of cytotoxic/cytostatic effects on MCF7 cells and in comparison to non tumor mouse fibroblasts they were tumor cell specific with selectivity index (SI) of 7.9 and 4.5 for KL1 and KL2, respectively (Table 2).

Compound KL3 turned out to be most active in induction of cytotoxic/cytostatic effects from all of three novel compounds. The IC50 values for tumor cells were form 0.5 μM to 10 μM. Only MCF7 breast cancer cells remained relatively resistant to KL3 with IC50 of 116 μM.

Such a good anti tumor profile of KL3 was accompanied with less toxicity towards NIH-3T3 cells in comparison to the parental PTOX.

As suspected – combination of two anti tumor agents within one molecule (KL3) improved the antitumor activity, in comparison to KL1 and KL2, and what is also very desiderated this drug was more toxic for most cancer cell lines than to no-tumor fibroblasts.

Table 1 Compilation of IC50 value of novel compounds and of the parental POTX estimated in 7 cell lines.
IC50 – Concentration of compound corresponding to 50% growth inhibition after 48-hour incubation.

	Inhibitory concentration (IC 50) [μM]						
Compound	Immortalized non tumorogenic cells	Tumor derived cells					
	Fibroblast	Cervix cancer	Breast cancer		Prostate cancer		Pancreas cancer
	NIH-3T3	HeLa	MDA-MB	MCF7	PC3	DU145	CFPAC
KL1	55	165	300	7	48	425	370
KL2	45	140	490	10	144	290	400
KL3	1	0.5	0.7	116	0.7	6.8	10
PTOX	0.5	0.5	125	123	10	1	100

The standard error bars did not extend 10% and for more transparency are not included in the table. Results are from 3 independent experiments.

The selectivity index is helpful for comparison between different tumor cell lines. However, when IC50 values are high, the comparison between substances does not matter. Obtained substances KL1 and KL2 are less toxic. Thus, we have obtained leading structures of lower cytotoxicity than parental PTOX. In turn, KL3 as compound represented by covalently bound of two anti tumor substances turned out to be the most effective with the highest anti tumor activity from all studied substances. The IC50 value for KL3 was below 1 μM for three cancer cell lines.

Table 2 Tumor cell selectivity

	Selectivity Index (SI)					
Compound	Tumor derived cells					
	Cervix cancer	Breast cancer		Prostate cancer		Pancreas cancer
	HeLa	MDA-MB	MCF7	PC3	DU145	CFPAC
KL1	0.3	0.2	7.9	1.1	0.1	0.1
KL2	0.3	0.1	4.5	0.3	0.2	0.1
KL3	2.0	1.4	0.009	1.4	0.1	0.1
PTOX	1.0	0.004	0.004	0.1	0.5	0.005

2.3. Microscopy analysis

A series of images was recorded and representative regions of particular groups are shown at Fig 1. Microscopic experiments were performed on NIH-3T3 cells conducted from disaggregated BALB/c mouse embryos. They are extremely sensitive to contact inhibition and are highly susceptible to transformation by SV40 VIRUS and murine sarcoma virus [60].

In comparison to the control group, demonstrating spindle-shaped morphology of NIH-3T3 cells that are elongated with long processes and share 95% of confluence, both treated groups vary in cell count and shape from the control group. Cells treated with 10 μM or 100 μM PTOX demonstrate 60 and 50% of confluence, respectively. In comparison to the controls, their processes are shortened and thickened, the perinuclear region is enlarged, the total surface occupied by a single cell is enlarged. We can observe also signs of cell degeneration like small rounded cells and pyknotic cells that's number increases by increasing concentration of PTOX.

Compound KL3 induced shortness of NIH-3T3 processes similarly to parental PTOX, also the perinuclear region was enlarged. Moreover, this enlargement was more pronounced than in PTX treated group. The ratio of degenerating cells was also higher than in PTX treated cells. We observed numerous pyknotic cells in KL3 100 μM treated group and some amount of big rounded cells – suggesting mitotic catastrophe as a reason of such perturbations. (Figure 1)

Even if such initial compounds might have diminished cytotoxic potencies compared with the parent PTOX, the ease of preparation of carefully designed libraries of analogues would lead to more informative studies and expeditious structure optimization. In this regard we have obtained less toxic scaffolds represented by KL1 and KL2 and a highly effective KL3 that is twice less toxic for NIH-3T3 cells in comparison to PTOX and turned out to be selective for tumor cells. To improve the molecules, further modifications should be designed and performed combined with studies on detailed mechanisms of action of these compounds.

3. Materials and methods

3.1. Synthesis

Methyl 4-(benzo[d][1,3]dioxol-5-yl)-5,6,7-trimethoxy-3-methyl-2-naphthoate (2) was prepared in accordance with the procedure previously described.3

(5R,6R)-Methyl 7-((S)-2-(hydroxymethyl) pyrrolidine-1-carbonyl)-5-(3,4,5-trimethoxy-phenyl)-5,6-dihydronaphtho[2,3-d][1,3]dioxole-6-carboxylate (3) was prepared in accordance with the procedure previously described.4

(5R,6R)-Methyl 7-(benzo[d]thiazol-2-yl)-5-(3,4,5-trimethoxyphenyl)-5,6-dihydronaphtho [2,3-d] [1,3]dioxole-6-carboxylate (4). A mixture of podophyllic aldehyde (prepared in accordance with the procedure previously described4) (64 mg, 0.15 mmol, 1 equiv.), 2-aminothiophenol (19.7 mg, 0.16 mmol, 1.05 equiv.) and Na2S2O5 (30 mg, 0.16 mmol, 1.05 equiv.) in 1 mL of DMSO was heated at 120˚C for 2 h. The reaction mixture was allowed to cool to room temperature, excess water was added, and yellow solid precipitate was collected by filtration. The precipitate was washed with water, dried and purified on a silica gel column, using ethyl acetate in n-hexane (gradient, from 0% to 10%) as an eluent. A yellow solid was obtained (52 mg, 0.098 mmol, 65%). M.p. 165–166°C. Rf (50% AcOEt/n-hexane) 0.54. [α]D25 = 281.0 (c 0.1, CHCl3). 1H NMR (CDCl3, 300 MHz): δ 7.95 (dd, J1 = 8.1 Hz, J2 = 0.6 Hz, 1H), 7.84 (dd, J1 = 7.8 Hz, J2 = 0.6 Hz, 1H), 7.43 (m, 2H), 7.35 (dt, J = 7.8, 1.2 Hz, 1H), 6.86 (s, 1H), 6.63 (s, 1H), 6.56 (s, 2H), 5.98 (d, J = 1.5 Hz, 1H), 5.96 (d, J = 1.5 Hz, 1H), 4.61 (d, J = 7.5 Hz, 1H), 4.44 (d, J = 7.8 Hz, 1H), 3.89 (s, 3H), 3.84 (s, 6H), 3.43 (s, 3H). 13C NMR (CDCl3, 75 MHz): δ 171.7, 167.1, 153.8, 153.3, 148.6, 146.5, 137.4, 134.8, 134.4, 133.4, 131.7, 128.5, 127.0, 126.2, 125.3, 123.1, 121.4, 108.8, 108.8, 106.6, 101.4, 77.4, 77.0, 76.6, 60.9, 56.1, 51.7, 49.1, 48.5. HRMS (ESI) m/z: calcd for C29H25NO7SH [M+H]+, 532.1424; found, 532.1440.

3.2. Reagents

PROX (SIGMA) and Novel compounds were dissolved in DMSO (SIGMA) at 100 mM and kept in the fridge until examination.

3.3. Cell Culture

Mouse NIH-3T3 fibroblasts (ATCC, USA) were cultured in Dulbeco (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), strepto-mycin 100 µg/ml.

For analysis of antitumor activity of examined compounds, following cell lines were used: MDA-MB-431, HeLa, PC3 and DU145. All of cell lines was obtained from ATCC. All cells were cultured in media recommended by suppliers. Then supplemented with standard antibiotics and 10% FCS all from Sigma-Aldrich. Cells were kept in 25 cm2 tissue flasks (Greiner, Berlin, Germany) and passaged every 2-3 days.

3.4. Cell Proliferation Assay

The cytotoxic/cytostatic effects of novel compounds on culture cells were examined in vitro using the crystal violet assay, as previously described (Mlynarczuk-Bialy et al., 2006). Briefly, cells (5 x 103 cells/well) were seeded in 96-well microtiter plates (BD, Biosciences, San Jose, California, USA) and incubated with serial dilutions of examined compounds. Inhibitors were added in quadruplicate to a final volume of 200 µL. Appropriate volumes of culture medium, supplemented with DMSO (<0.1%) were added as controls. After an incubation period of 24, 48 or 72 hours, cells were washed once with PBS, fixed with 70% ethanol for 30 min and finally stained with 0,1% crystal violet in PBS for 30 min and washed carefully with water to remove unbound dye. The remaining dye was eluted by 1% SDS in water and determined at 550 nM. Cytostatic/cytotoxic effect was expressed as relative viability of treated cells (% of control cells incubated with medium only) and was calculated as follows: relative viability = (Ae - Ab) x 100/(Ac-Ab), where Ab is background absorbance, Ae is experimental absorbance and Ac is the absorbance of untreated controls.

3.5. Light microscopy

In order to assess the influence of novel derivates on morphology of examined cells, after 48 h of incubation period, cells form cell culture were directly imaged by a phase contrast microscope (Juli, NanoEnTek) at magnification of 100x.

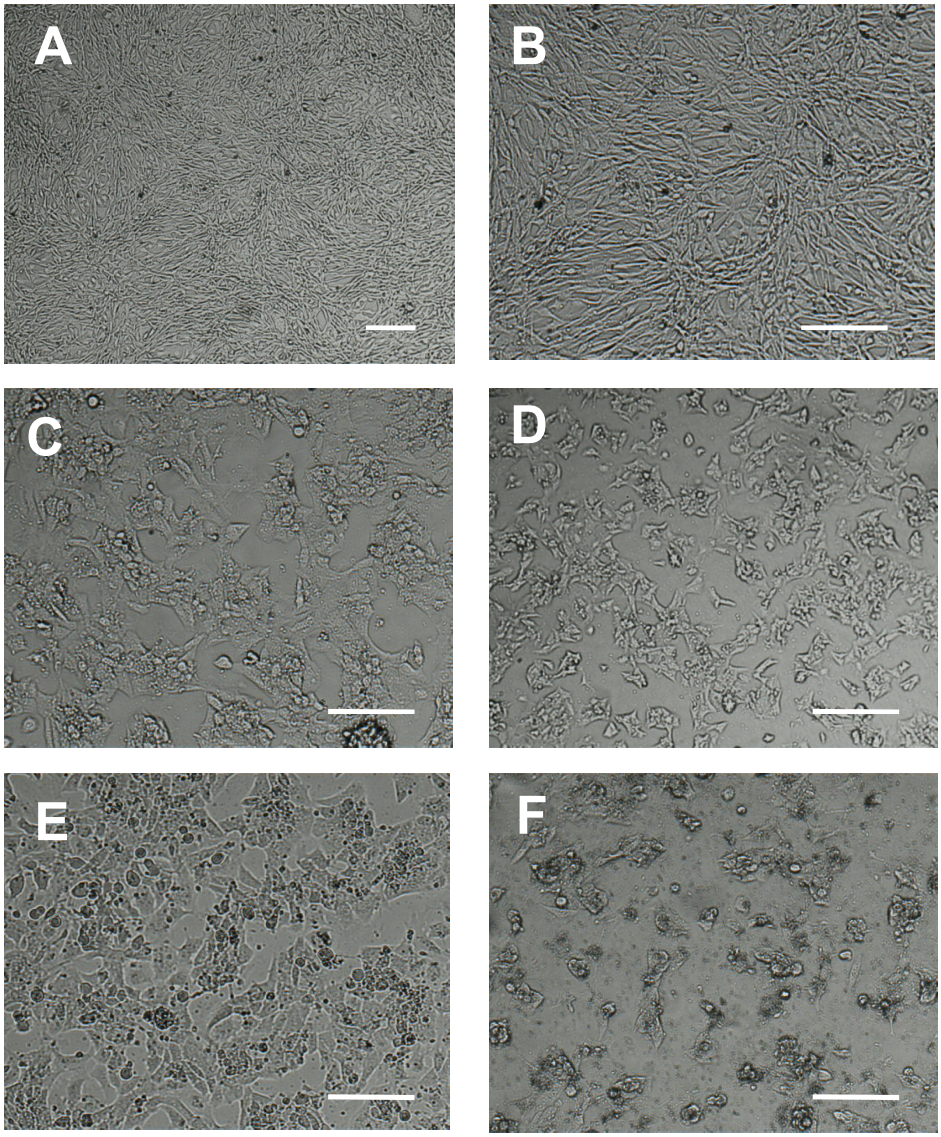


Figure 1: Photomicrographs of NIH-3T3 cells:
Untreated cells (A, B smaller and bigger magnification, respectively), C,D: cells treated with PTOX 10 μM or 100 μM–respectively, or E,F groups treated with KL3 10 μM or 100 μM –respectively. Scale bars 50 μM

In comparison to the controls (A,B) we observe dose-dependent cytopathic effects including shortness of cytoplasmic processes and thickness of perinuclear region. In the higher drug concentration, we observe also cell rounding and detachment from the bottom. In comparison to PTOX (C,D) the effects induced by KL3 (E,F) are similar but more pronounced (advanced).

4. Summary and conclusion

We aimed to create novel compounds of anti-cancer drug – PTOX. We planned and designed the synthesis of more effective as well as less toxic compounds. Furthermore, we were also focused on economic aspects and would like to reduce costs of synthesis. Due to these assumptions we planned to use cheap and easily available substrates. Moreover, in the process of synthesizing we don't need to use heavy metal catalysts, and thus there is no risk of conta-

mination of compounds obtained with heavy metals (whose content in the drug must be at a very low level). The next advantage form the used methodology, thanks to the use of flow conditions in the photocyclic process, it was easy to conduct synthesis on a large scale. This methodology also allowed obtaining numerous derivatives with differently decorated rings, thanks to which it was possible to influence parameters such as: solubility, bioavailability, stability. What is interesting, when it comes to the KL3 compound itself, it was the first synthesis of the PTOX and benzothiazole congeners with the cis configuration at the C1 and C2 carbon atoms.

For in vitro tests of new substances, we applied low-cost and well-standardized viability assays in combination with bio-imaging of the tested cells. We showed that the applied methods are able to verify in an unambiguous manner whether the examined chemical structures exhibit biological effects.

Result of cytotoxicity assay and microscopic analysis proved, novel compounds are less toxic and more effective in comparison to parental PTOX. That is a promising result because the toxicity of PTOX excludes its systemic application. Anti cancer KL3 compound with better effectiveness and lover toxicity can be useful in more applications as the initial substances.

In summary, we obtained KL3 derivative, that is less toxic than the parental one, in an innovative synthesis process that allows to synthesize large quantities of the product in its pure form – this work is therefore a comprehensive description of the compound in cell culture-based model. Its future testing in animal models can verify pre-clinical potential of KL3 compound.

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